Supporting Information

Mattison et al. 10.1073/pnas.1102187108

SI Materials and Methods

Animals and Animal Care. The test subjects weighing 2.5 to 4.2 kg were received into quarantine at an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility maintained on a 12-h:12-h light:dark cycle, at a temperature of 23 to 29 °C, humidity of 30% to 70%, and 10 to 15 air exchanges per hour. The animals were housed individually, provided with water ad libitum, and were fed High Protein Monkey Diet Jumbo (#5047, PMI Nutrition International) daily, supplemented with children's vitamins and fresh fruit three times a week. The monkeys were antibody-negative for simian type D retrovirus, simian immunodeficiency virus, and simian T-cell leukemia virus, and were released from a 1-mo quarantine period following three negative tuberculosis tests and veterinary health assessment indicating good health. Once released from quarantine, the test subjects underwent a 4-mo acclimation and training period in the Primate Facility before the initiation of methylphenidate hydrochloride (MPH) dosing at ~25 to 28 mo of age.

All test subjects on the study underwent genetic safety assessments and behavioral testing during the dosing period, and the data have been reported elsewhere (1, 2). Banana-flavored reward pellets (Bioserv) were used as reinforcers in the behavioral studies. Both the primate biscuits and the reward pellets were lot-tested before use to confirm that the dietary constituents (e.g., fat, protein, and vitamins) were within the acceptable range and that contaminant levels—including heavy metals, fumonisin, aflatoxin, and pesticides—were below maximum acceptable levels. Test subject diets were formulated on an individual basis such that the rate of body weight gain in an individual nonhuman primate (NHP) was at 0.1 kg per month in the initial phases of the experiment. The rate of gain was increased to 0.2 kg per month as the animals approached puberty.

Dose Selection. Animals were assigned to dose groups by a randomized weight ranking. Weights were obtained 2 wk before the scheduled start of dosing. Thirty consecutive numbers were assigned to the monkeys according to their body weights. The monkeys were allocated to three treatment groups consecutively by randomly generating the group numbers. The animals assigned to the control group weighed between 2.8 and 4.2 kg, the low-dose group animals weighed between 2.9 and 4.2 kg, and those assigned to the high-dose group weighed between 3.0 and 4.5 kg.

It should be noted that the initial dose selection in the juvenile monkeys was based on two previous studies in adult monkeys. The first study was a pharmacokinetics (PK) study (3) and the second was a preliminary study that we conducted to determine the most appropriate vehicle for MPH. In the latter study, doses of 0.15 mg/kg of MPH dispersed in Prang resulted in peak plasma levels of 2.0 to 5.0 ng /mL in aged, female NHP. This level closely approximated the target pediatric blood level of 2 to 10 ng/mL described in Swanson and Volkow (4) and was selected as the low dose for the study. A 10-fold increase in the dose (1.5 mg/kg) was selected as the high dose to provide a broad dose range for the genetic and behavioral toxicity studies (1). However, it was subsequently observed that this dosing schedule produced inadequate serum concentrations, requiring an increase in dose to produce clinically relevant concentrations.

Exposure Assessment. Labeled internal standard (100 μL of 1 ng/mL in 0.1% formic acid) was added to each thawed plasma sample (10 μL) and 800 μL 0.1% formic acid. Samples were purified using solid phase extraction in 96-well plates and analyzed using electrospray tandem mass spectrometry (LC-ES-MS/MS) in the multiple-reaction monitoring mode by monitoring specific transitions for labeled and unlabeled MPH and unlabeled ritalinic acid (RA, the major metabolite of MPH in NHP) using the procedures previously published (3). Quantitative accuracy ranged from 95% to 111% and the inter- and intraday method precision ranged from 2% to 6% relative SD. The limits of quantification were 0.1 ng/mL for MPH and 0.03 ng/mL for RA and the limits of detection for MPH and RA were 0.03 and 0.009 ng/mL, respectively.

Crown-Rump Measurements. Crown-rump measurements (cm) were performed monthly from month 17 through month 41. This measurement was made using a stadiometer as the test subject was chaired. The base of the instrument was placed under the ischial callosities and the top plate was placed on top of the test subject's head.

Endocrine Analyses. Monthly serum samples were assayed for total testosterone using a solid-phase RIA (RIA; Coat-A-Count, Siemens), for inhibin B using an ELISA (DSL-10–8410; Diagnostic System Labs), for primate leptin using a RIA (PL-84K; Linco Research), and for FSH using an RIA as described in Ramaswamy et al. (5). Intra- and interassay coefficient of variations, respectively, were 17.6% and 7.3% for testosterone, 5.6% and 6.2% for inhibin B, <7% and 4% for FSH, and 8.4% and 10.0% for leptin. Sensitivity was 0.04 ng/mL for testosterone, 7 pg/mL for inhibin B, 0.05 ng/mL for FSH, and 0.2 ng/mL for leptin. The testosterone, inhibin B, and leptin assays were performed by Toxicologic Pathology Associates in Jefferson, AR. The FSH assays were conducted by the Assay Core of the Specialized Cooperative Centers Program in Reproduction and Infertility Research at the University of Pittsburgh School of Medicine. The diurnal serum samples were assayed for luteinizing hormone (LH) using a homologous (macaque) RIA (6) and for total testosterone using the solid-phase RIA (Siemens) as described in Ramaswamy et al. (7). Intra- and interassay coefficients of variation were < 7% for both LH and testosterone. Sensitivity was 0.12 ng/mL for LH and 0.04 ng/mL for testosterone. These analyses were conducted by the Assay Core of the Pittsburgh Specialized Cooperative Centers Program in Infertility and Reproduction Research.

Semen Collection. At each time, semen collection was attempted from each test subject on four occasions within a 2-wk period, with a minimum of 1 d of rest between collection attempts. Semen collections were attempted in the normal restraint chairs used for blood collection, with additional restraint provided by the animal-care staff, if necessary, to minimize stress on the test subject.

Semen was collected using penile electroejaculation. Initially, the penis was massaged from the sheath, followed by the use of a small vibrator for penile stimulation. Two strips (4 × 0.5 cm) of nonmetallic cardiac defibrillation pads were prepared and kept moist. One strip was wrapped around the base of the penis and the other just below the glans. The two electrodes from the Grass S88 stimulator were attached to the pad strips. The stimulator settings were 20 pulses per second, 18-ms duration, and 20-ms delay. The voltage was varied from 0 to 50 V (50 mAmp). Stimulation was applied at the lowest voltage and gradually increased over ~90 s.

A warm (37 °C) glass scintillation vial with 2 mL of PBS was used to collect the semen. The head of the penis was held inside the vial opening to catch the entire ejaculate, which was immediately transported to the laboratory. The sample was viewed for the presence of a copulatory plug, which was removed from

the specimen, if present. If no ejaculation occurred the animal was allowed to rest for approximately 1 min and the stimulation was repeated. If no ejaculation occurred within 10 min, the animal was returned to his cage.

Semen Evaluation. Sperm viability was determined by hypo-osmotic swelling (HOS assay). Sperm motility and concentrations were measured using the computer-assisted sperm analysis system (HTM-IVOS, Hamilton-Thorn).

Sperm Morphometry. Sperm morphometry was conducted on one slide per animal providing a semen sample for each collection period. One hundred sperm per slide were analyzed using the Metric program of the HTM-IVOS System (Hamilton-Thorn).

Sperm Morphology. Sperm morphology was conducted on one slide per animal providing a semen sample for each collection period. Sperm morphology was evaluated using the strict criteria (8) for human sperm morphology and the traditional human morphology scheme (9), modified to include the forms noted in initial evaluation of the NHP slides. The abnormal sperm classifications were:

Head abnormalities: bicephalic, tapering, pyriform, abnormal head size, amorphous head, ridged head, elongated head, pointed head;
Neck and midpiece abnormalities: neck/midpiece defect, thick midpiece, bulged midpiece, bent neck; and
Tail defects: missing tail, tail defect, hairpin tail, coiled tail, double tail, cytoplasmic droplet.

In addition to the defects listed above, the number of white blood cells per 100 sperm and the number of immature germ cells per 100 sperm were calculated. Two-hundred sperm were classified, except for samples with a low sperm concentration ($n = 5$), where only 100 sperm were analyzed per sample.

SI Results

Body Weight. Each test subject was weighed five times per week throughout the 40-mo dosing period. Individual weight gain was evaluated monthly and adjustments to the diet were made as necessary to maintain a similar rate of gain in all animals. Dose-related effects on performance in behavior testing were noted and attributed to the anorexic effect of MPH (2). However, test subjects were given 24-h availability to their daily ration, which precluded an effect on weight gain. To evaluate the effect of dose and length of treatment on body weight, the weight for each animal on the same date each month was analyzed by ANOVA. A significant increase ($P < 0.0001$) in body weight was detected during the 40-mo exposure period. However, neither the ANOVA nor the Dunnett's test revealed a significant effect of dose on body weight.

Exposure Assessment. In designing this study, the initial dose for the low-dose group was 0.15 mg/kg, which was expected to produce plasma concentrations similar to those observed in humans (2–20 ng/mL). The dose for the high-dose group was 1.5 mg/kg, which was expected to result in plasma concentrations 5- to 10-times higher (20–200 ng/mL) than observed clinically. However, plasma concentrations of MPH were substantially lower than expected for the first 4 mo of the study; consequently, doses for both the low- and high-dose groups were slowly increased to 2.5 mg/kg twice a day and 12.5 mg/kg twice a day, respectively. Treatment with these higher doses resulted in plasma levels that were ~10 and 100 ng/mL for the low- (Fig. S1, *Upper*) and high-dose (Fig. S1, *Lower*) groups, respectively. The concentrations in the low-dose group were similar to the clinical range of 2 to 20 ng/mL (4). Plasma levels in the high-dose group were 5- to 10-fold higher than the therapeutic range. The data for month 0 through month 30 has previously been published in Morris et al. (1).

As noted above, plasma levels of MPH were lower than expected and were raised to achieve the appropriate levels. PK experiments were conducted at quarterly intervals over a 2-y dosing period and are presently undergoing analysis. This analysis may help to determine if the lower levels observed in this cohort are because of a lower rate of absorption in the intestine or a difference in first-pass metabolism in the aged animals used in the preliminary study.

Crown-Rump Measurements. ANOVA revealed a significant increase ($P < 0.0001$) in the crown-rump measurement over the course of the experiment. No significant effect of dose was detected either overall ($P = 0.61$) or for any individual time point. No significant differences between the control and low-dose groups or between the control and the high-dose groups were detected either overall or at any time point.

Endocrine Measurements. ***Luteinizing hormone.*** The diurnal pattern of serum LH concentrations was also assessed at month 35 (Fig. S3, *Upper*) and Month 38 (Fig. S3, *Lower*). No effect of dose on serum LH levels was found at any time point ($P > 0.05$). A significant diurnal effect was found at month 35 ($P = 0.0002$), but not at month 38 ($P = 0.1688$).
FSH levels. Significant differences ($P = 0.0084$) in serum FSH levels were detected as the animals matured, but no consistent overall effect of dose was observed (Fig. S4).

Semen Analysis. No significant differences were found in the sperm concentration from month 27 through month 38 of treatment. Significant differences between the control and high-dose groups in the percentage of sperm with elongated heads ($P = 0.0019$) were found only at month 27 (Fig. S5). Morphometric analysis of elongation factor (width/length × 100) indicated that the average elongation factor was significantly reduced ($P = 0.0123$) in the high-dose group at month 27 (Fig. S6). A significant interaction between dose and time was found in the percentage of sperm with elongated heads ($P = 0.0459$) and in the percentage of sperm with reduced elongation factor ($P = 0.0103$).

1. Morris SM, et al. (2009) The genetic toxicology of methylphenidate hydrochloride in non-human primates. *Mutat Res* 673(1):59–66.
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3. Doerge DR, Fogle CM, Paule MG, McCullagh M, Bajic S (2000) Analysis of methylphenidate and its metabolite ritalinic acid in monkey plasma by liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* 14:619–623.
4. Swanson JM, Volkow ND (2003) Serum and brain concentrations of methylphenidate: Implications for use and abuse. *Neurosci Biobehav Rev* 27:615–621.
5. Ramaswamy S, Pohl CR, McNeilly AS, Winters SJ, Plant TM (1998) The time course of follicle-stimulating hormone suppression by recombinant human inhibin A in the adult male rhesus monkey (*Macaca mulatta*). *Endocrinology* 139:3409–3415.
6. El Majdoubi M, Ramaswamy S, Sahu A, Plant TM; S6 (2000) Effects of orchidectomy on levels of the mRNAs encoding gonadotropin-releasing hormone and other hypothalamic peptides in the adult male rhesus monkey (*Macaca mulatta*). *J Neuroendocrinol* 12(2):167–176.
7. Ramaswamy S (2005) Pubertal augmentation in juvenile rhesus monkey testosterone production induced by invariant gonadotropin stimulation is inhibited by estrogen. *J Clin Endocrinol Metab* 90:5866–5875.
8. World Health Organization (1992) *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction,* 3rd ed. (Cambridge University Press, Cambridge).
9. World Health Organization (1999) *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction,* 4th ed. (Cambridge University Press, Cambridge).

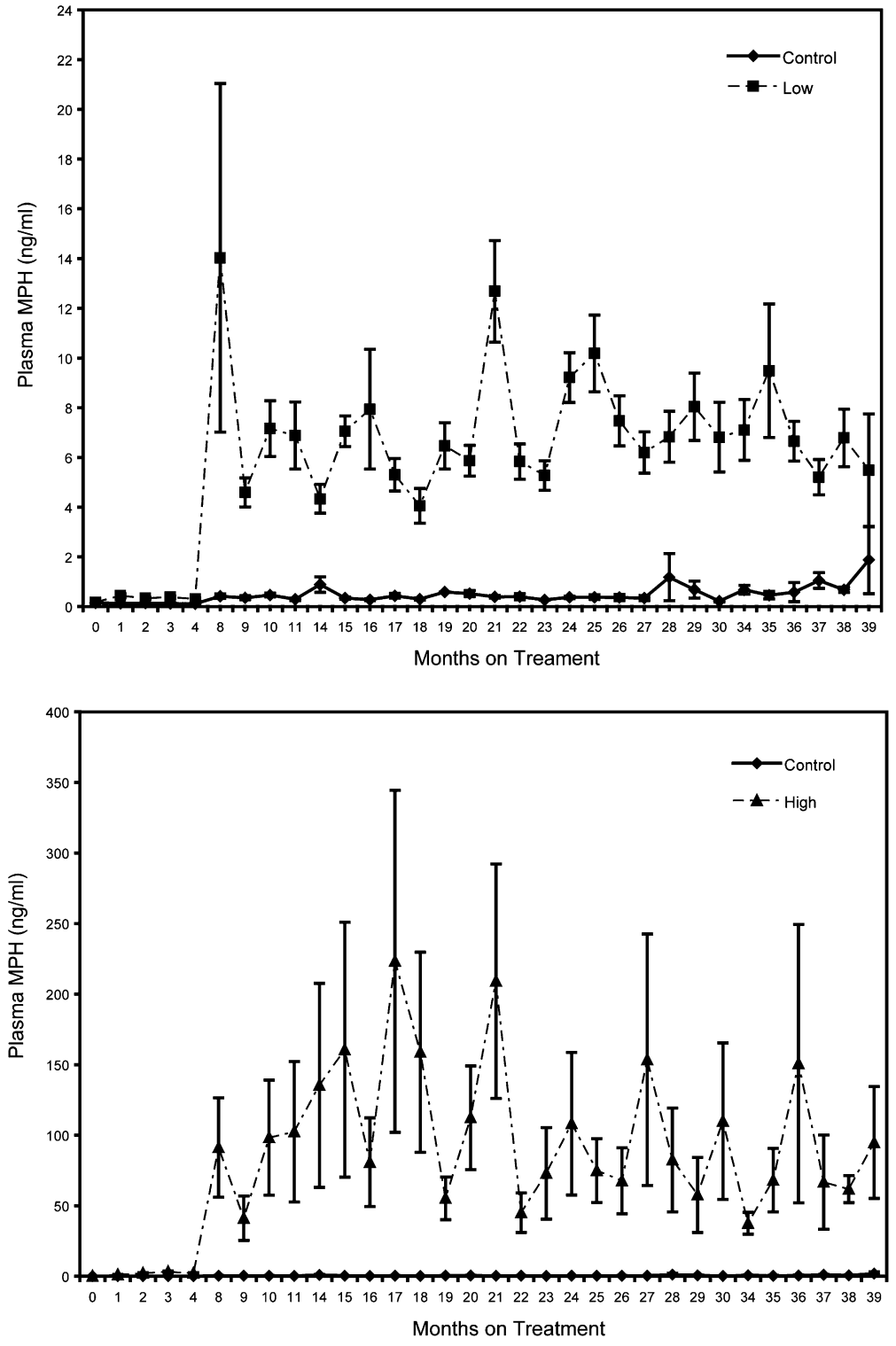

Fig. S1. Mean monthly plasma MPH levels (± SEM) in peripubertal male rhesus monkeys chronically exposed to the low (*Upper*) or high (*Lower*) dose of MPH. Plasma levels of MPH were determined by LC-ES-MS-MS.

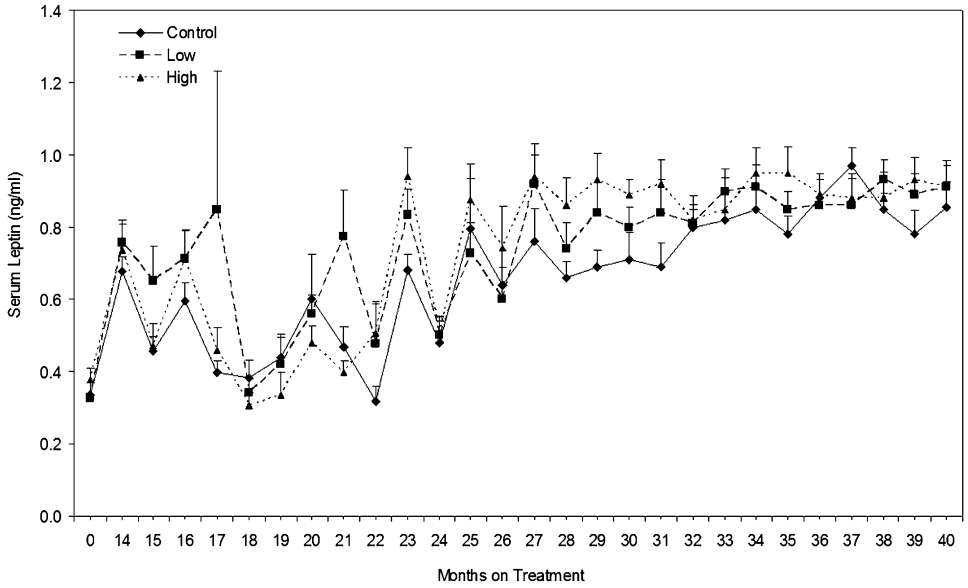

Fig. S2. Effect of dose and length of treatment on mean serum leptin concentrations (± SEM) in peripubertal male rhesus monkeys chronically exposed to MPH. A 3- to 4-mo peak in serum leptin levels occurred initially, followed by a trough, and then a progressive increase ($P < 0.0001$) as the monkeys matured. A significant ($P = 0.0188$) overall effect of dose on serum leptin levels was detected by ANOVA. Serum leptin levels were higher in the low- and high-dose groups than in the control group ($P = 0.0212$, $P = 0.0316$, respectively).

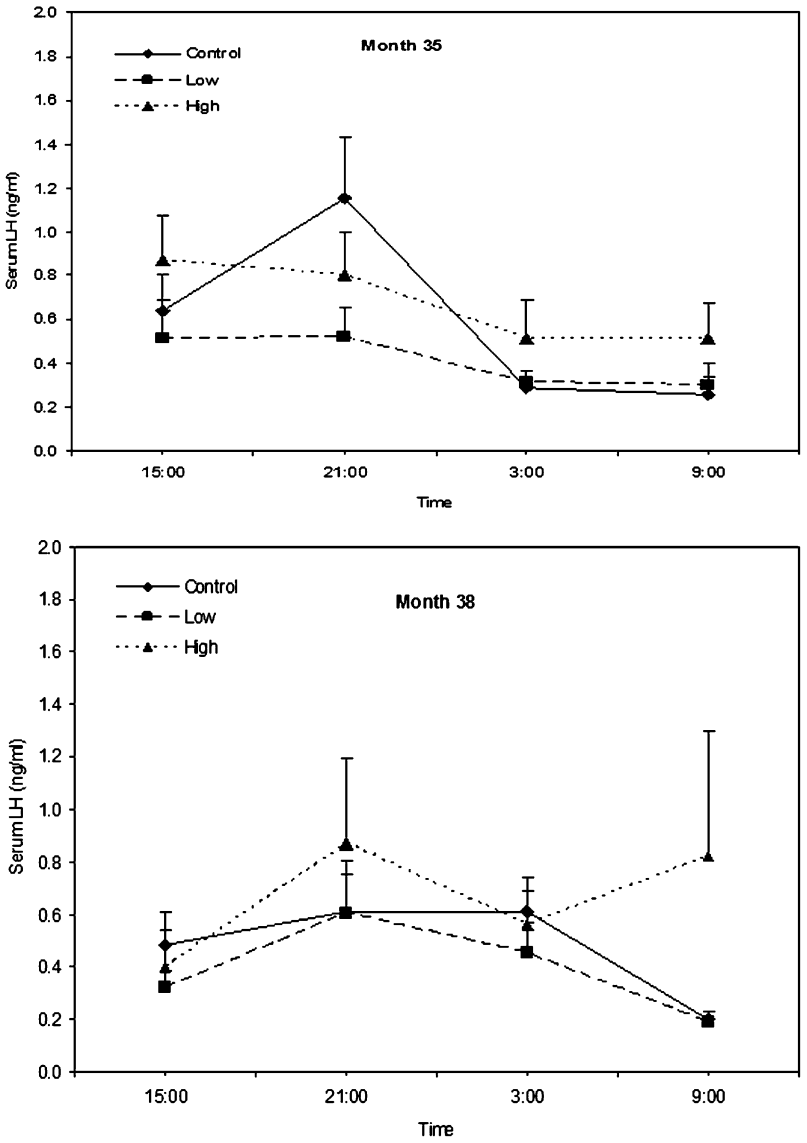

Fig. S3. Effect of dose and time of day on mean serum LH levels (± SEM) in male rhesus monkeys chronically exposed to MPH. Samples were collected at 1500, 2100, 0300, and 0900 hours. Significant time effects were detected ($P = 0.0002$) at month 35 (*Upper*), but not at month 38 (*Lower*). No effect of dose ($P > 0.05$) on serum LH levels was found at either time point.

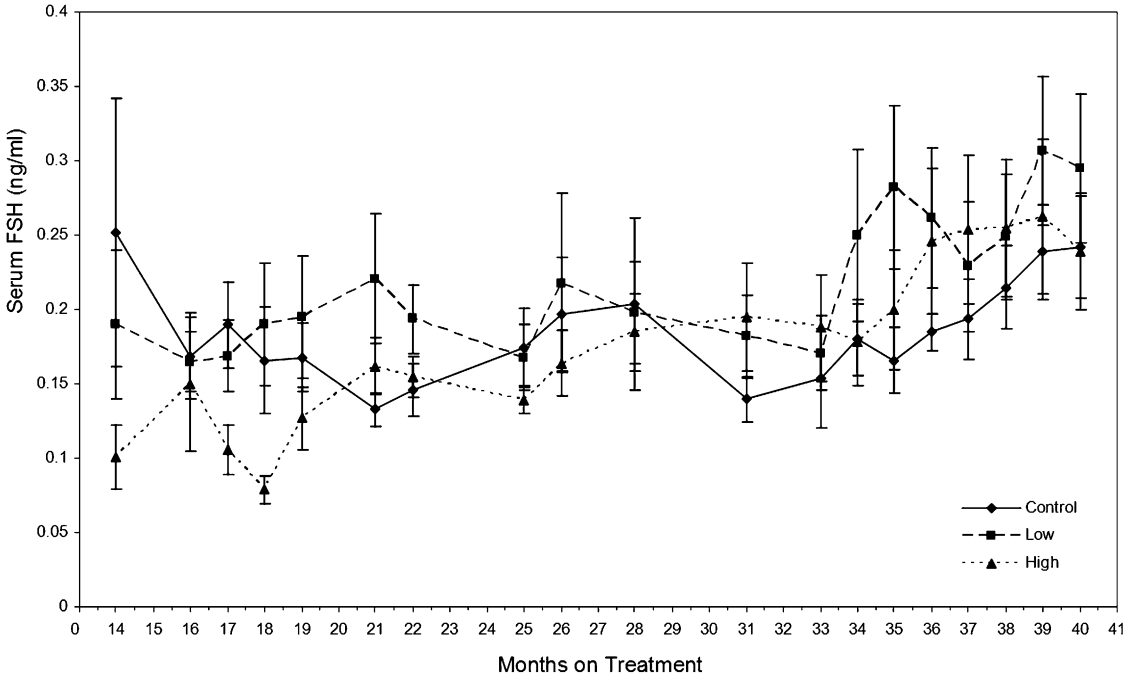

Fig. S4. Effect of dose and length of treatment on mean serum FSH concentrations (± SEM) in peripubertal male rhesus monkeys chronically exposed to MPH. Although serum FSH levels increased ($P = 0.0084$) as the animals matured, no consistent effect of dose was detected by either ANOVA or the Dunnett's test.

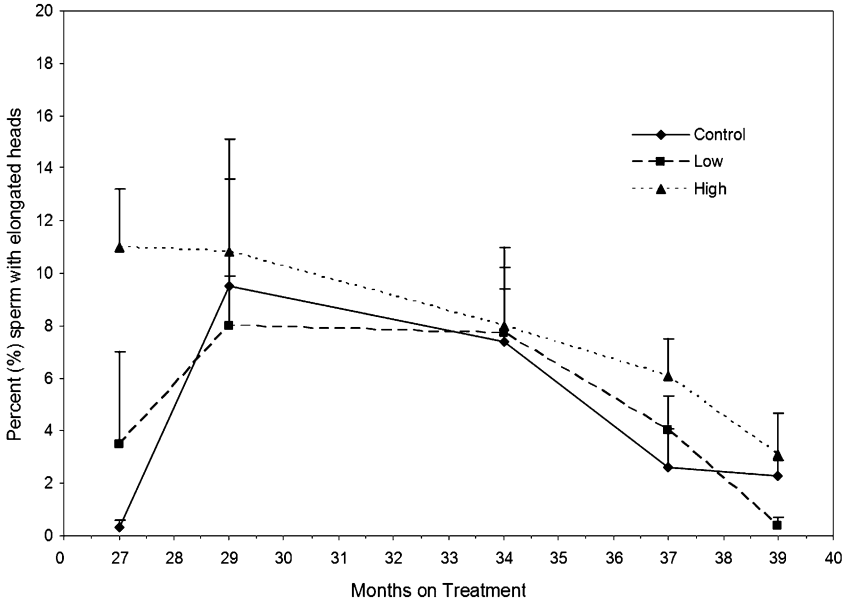

Fig. S5. Effect of dose on the mean percentage of sperm with elongated heads (± SEM) in peripubertal male rhesus monkeys chronically exposed to MPH. At month 27, a significant increase ($P = 0.0019$) in the percentage of sperm with elongated heads. No effect of the low dose on the percentage of abnormal sperm was detected.

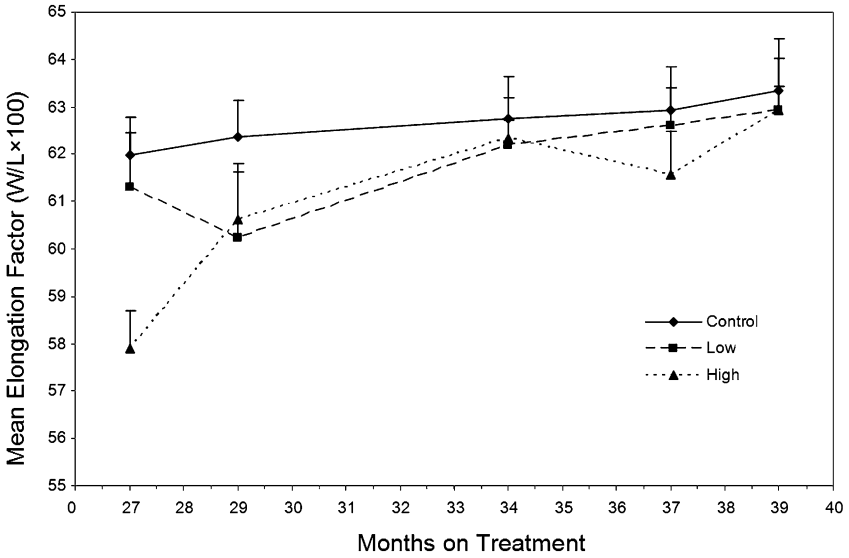

Fig. S6. Effect of dose on the mean elongation factor of sperm (± SEM) obtained from peripubertal male rhesus monkeys chronically exposed to MPH. At month 27, a significant decrease ($P = 0.0123$) in the mean elongation factor (width/length × 100) was found in the sperm obtained from the high-dose group animals. No effect of the low dose on this measurement was detected.